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EXAMINER	
WOITACH, JOSEPH T	
ART UNIT	**PAPER NUMBER**
1632	

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Please find below and/or attached an Office communication concerning this application or proceeding.

Interview Summary	Application No.	Applicant(s)	
	09/982,637	THOMSON, JAMES A.	
	Examiner	Art Unit	
	Joseph T. Woitach	1632	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Joseph T. Woitach*.

(2) *Nicholas J. Seay*.

(3)*James A. Thomson*.

(4)*Carl Gulbrandsen, Paulanne Chelf, Elizabeth Donley*.

Date of Interview: *19 October 2004*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☒ Yes e)☐ No.
If Yes, brief description: *comments and exhibits for discussion were provided (see attached)*.

Claim(s) discussed: *pending claims*.

Identification of prior art discussed: *Labosky et al. 1994 (attached)*.

Agreement with respect to the claims f)☒ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *See Continuation Sheet*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicants noted that a terminal disclaimer would be filed to obviate the remaining rejection of record. Regarding the request for an interference with the instant application and 08/813,829, Applicants noted that the '829 application was suspended for consideration of the request. Applicants argued that the instant application should not be included in the interference because the cells disclosed by the two applications are different. First, iti was noted that the instant application (and parent applications) uses starting materials and methods that are different from that of '829 (providing declarations and prosecution history from applications related to '829). As a result of the different methods and materials disclosed, it was argued the the cells of '829 are EG cells, not ES cells as instantly claimed. In durhter support Labosky et al. (1994) was provided to differentiate the two cell types and demonstrate further support for the interpretation of the '829 disclosure. finally it was noted that the claims of '829 can be distinguished from that of the instant claims because the instantly claimed cells do not express SSEA-1, wereas '829 specifically claims that the cells comprise this marker. In addition, it was noted that this further supports the interpretation that the cells of '819 are EG cells. Examiner agreed that the claimed cells disclosed in the two applications are different, in particular with respect to the distinguishing markers present on the cell (both disclosed in the specification and specifically claimed). Moreover, Examiner agreed that starting materials disclosed in the two applications are different, and most likely the reason giving rise to the difference in cell surface markers of the two different claimed products. Examiner indicated that he would enter Applicants comments and Exhibits with the instant interview summary, and give them full consideration in the instant application.

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Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (*Igf2r*) gene compared with embryonic stem (ES) cell lines

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SUMMARY

Primordial germ cells of the mouse cultured on feeder layers with leukemia inhibitory factor, Steel factor and basic fibroblast growth factor give rise to cells that resemble undifferentiated blastocyst-derived embryonic stem cells. These primordial germ cell-derived embryonic germ cells can be induced to differentiate extensively in culture, form teratocarcinomas when injected into nude mice and contribute to chimeras when injected into host blastocysts. Here, we report the derivation of multiple embryonic germ cell lines from 8.5 days post coitum embryos of C57BL/6 inbred mice. Four independent embryonic germ cell lines with normal male karyotypes have formed chimeras when injected into BALB/c host blastocysts and two of these lines have transmitted coat color markers through the germline. We also show that pluripotent cell lines capable of forming teratocarcinomas and coat color chimeras can be established from primordial germ cells of 8.0 days p.c. embryos and 12.5 days p.c. genital ridges. We have examined the methylation status of the putative imprinting box of the insulin-like growth factor type 2 receptor gene (*Igf2r*) in these embryonic germ cell lines. No correlation was found between methylation pattern and germline competence. A significant difference was observed between embryonic stem cell and embryonic germ cell lines in their ability to maintain the methylation imprint of the *Igf2r* gene in culture. This may illustrate a fundamental difference between these two cell types.

Key words: primordial germ cells, embryonic germ cells, embryonic stem cells, imprinting, growth factors, insulin-like growth factor type 2 receptor gene (*Igf2r*), methylation

INTRODUCTION

Cells that give rise to the primordial germ cells (PGCs) of the mouse are first identified by lineage tracing as a population of cells within the proximal margin of the 6.5 days post coitum (p.c.) epiblast, close to the junction with the extraembryonic ectoderm (Lawson and Hage, 1994). By 7.5 days p.c. PGCs have migrated to the extraembryonic mesoderm at the posterior of the primitive streak near the base of the allantois where they can be identified by staining for endogenous alkaline phosphatase (AP) (Ginsburg et al., 1990). The PGCs then become associated with the hindgut endoderm and migrate through the gut mesentery and begin to arrive at the genital ridges by 10.5 days p.c. During the process of moving from the posterior primitive streak to the genital ridges PGCs increase in number from about 150 cells at 8.5 days p.c. to approximately 25,000 at 13.5 days p.c. (Tam and Snow, 1981). By 13.5 days p.c. PGCs within the genital ridge cease dividing; those in the female enter meiosis and those in the male undergo mitotic arrest (Ginsburg et al., 1990).

Given the importance of PGCs to the continuation of the species, it is of interest to define the in vivo factors that control their proliferation, survival and migration. Progress has come from the study of mouse mutants, such as Dominant White-spotting (*W*) and Steel (*Sl*), in which PGCs do not follow their normal tightly regulated pattern of proliferation and differentiation, resulting in severe defects in fertility in homozygotes (Mintz and Russell, 1957; McCoshen and McCallion, 1975). The finding that *W* and *Sl* encode, respectively, the *c-kit* tyrosine kinase transmembrane receptor (Chabot et al., 1988; Geissler et al., 1988) and its ligand, Steel factor (SF, also known as stem cell factor, mast cell growth factor and c-kit-ligand), (Williams et al., 1990; Flanagan and Leder, 1990; Zsebo et al., 1990; Huang et al., 1990,) suggests that polypeptide growth factors, their receptors and downstream intracellular signalling pathways play key roles in regulating the proliferation, migration and differentiation of PGCs.

Previous work from our laboratory (Matsui et al., 1991,1992) and others (Dolci et al., 1991; Godin et al., 1991; Resnick et al., 1992) has shown that specific soluble and membrane-associated polypeptide growth factors are necessary for PGCs to survive and proliferate in culture. When PGCs from 8.5 days p.c. embryos are cultured with a mixture of membrane associated and soluble SF, leukemia inhibitory

factor (LIF) and basic fibroblast growth factor (bFGF), PGCs continue to divide in culture and give rise to lines of undifferentiated cells. These cells have been termed embryonic germ cells or EG cells to distinguish them from blastocyst-derived embryonic stem (ES) cells (Resnick et al., 1992). Once established, these EG cells can proliferate without added FGF and SF, but they still require feeder cells and serum factors. EG cells can differentiate into embryoid bodies in vitro and form teratocarcinomas in vivo. Recent studies have shown that EG cell lines derived from 129/Sv 8.5 days p.c. PGCs can contribute to the germline of chimeras (Stewart et al., 1994) and we show here that the same is true for EG cell lines derived from C57BL/6 PGCs.

One of the events associated with germ cell differentiation is a change in the methylation status of parentally imprinted genes (Barlow, 1993). Because it is not feasible to isolate large numbers of primordial germ cells from very early embryos, we have studied EG cell lines established from PGCs at various times during development in the hope of gaining some information about the imprinting process in vivo. We have analyzed the methylation state of the putative imprinting box of the maternally expressed insulin-like growth factor 2 receptor (*Igf2r*) gene (Stöger et al., 1993) in different EG cell lines and show that the methylation of region 2 of the *Igf2r* gene in most EG cell lines differs from that characteristic of normal somatic cells and ES cells. Moreover, in one cell line this methylation is lost in culture. However, there appears to be no correlation between the methylation pattern and the ability of the cells to contribute to the germline of chimeric mice. This illustrates a difference between ES cells and EG cells, a distinction that may reflect the difference in the developmental program of an inner cell mass cell versus a PGC.

MATERIALS AND METHODS

Mouse embryos and cell lines

C57BL/6, BALB/c, Swiss Webster, ICR, (C57BL/6 × DBA)F_1 and nude mice were obtained from Jackson Labs, Harlan Labs or Taconic Farms. Noon on the day of plug is 0.5 days post coitum (p.c.).

The ES cell lines tested were all derived from 129/Sv mice. Cell lines D3, J1 and R1 were kindly provided by Drs Thomas Doetschman, Rudolph Jaenisch and Janet Rossant, respectively. SC6 was derived from AB-1 cells obtained from Dr Allan Bradley. TL1 was derived in this laboratory.

Tumors were initiated in nude mice by the subcutaneous injection of 1×10^6 cells at each of four locations on the back. Tumors were dissected and processed for histological analysis as described (Matsui et al., 1992).

PGC culture

Cultures were initiated as described (Matsui et al., 1992) by dissecting C57BL/6 8.5 days p.c. embryos free of extraembryonic tissues. Fragments comprising the posterior third of the embryo (from the base of the allantois to the first somite) were then pooled, rinsed with Dulbecco's Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) and dissociated with 0.25% trypsin, 1 mM EDTA (GIBCO) and gentle pipetting. This single cell suspension is then plated in 0.1% gelatin-coated 24-well dishes (Corning) with irradiated *Sl/Sl*^{d} m220 cells as feeder layers at a concentration of approximately 0.5 embryo equivalents per well. The cultures were grown in Dulbecco's modified Eagle's medium (DMEM) (Specialty Media, Lavallette, NJ) supplemented with 4.5 g/l glucose, 0.01 mM non-essential amino acids (GIBCO), 2 mM glutamine (GIBCO), 50 μg/ml gentamycin (Sigma), 15% fetal bovine serum (selected batches, Hyclone) and 0.1 mM 2-mercaptoethanol (Sigma). For these primary cultures, the medium was additionally supplemented with soluble recombinant rat SF at 60 ng/ml, bFGF at 20 ng/ml (GIBCO) and LIF at 20 ng/ml. After 6 days some of the cultures are stained for alkaline phosphatase (AP) as described (Matsui et al., 1992) in order to assess the survival and proliferation of PGCs. After 10 days, parallel cultures are dissociated into single cells and plated onto mouse embryo fibroblast (mef) feeder layers with LIF (ESGRO, GIBCO 1000 U/ml). These cultures were monitored for the appearance of colonies of EG cells. Individual EG colonies were isolated with a micropipette and lines established. EG cultures were then maintained in the same manner as ES cell lines with irradiated mefs as feeder cells and LIF (Smith et al., 1988; Williams et al., 1988). Cultures from 12.5 days p.c. genital ridges or 8.0 days p.c. embryos were initiated and maintained in a similar manner.

Blastocyst injection

All EG cell lines were karyotyped (Robertson, 1987) and only lines with 80% or more of the cells containing 40 chromosomes were used for blastocyst injection. Ten to twenty EG cells at passage numbers 6 to 10 were injected into 3.5 days p.c. blastocysts from BALB/c mice. Foster mothers were (C57BL/6 × DBA)F_1 females mated to vasectomized Swiss Webster males. Injected blastocysts were transferred to the uterus of 2.5 days p.c. foster mothers (Hogan et al., 1986) and chimeric pups were identified by their coat color. Chimeras were bred to either BALB/c or ICR mice and germline transmission was judged on the day of birth by the presence of eye pigment.

Southern analysis

EG cell lines from passage number 6 to 10 were cultured without feeder layers on gelatin-coated 6 cm tissue culture dishes (Corning) for at least two passages in order to remove contaminating feeder cells while differentiation was inhibited by the continued presence of LIF in the medium. Cultures were dissociated with 0.25% trypsin, 1 mM EDTA, washed 3× with Dulbecco's Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) and lysed in TENS (100 mM EDTA, 50 mM Tris-Hcl pH8.0, 100 mM NaCl) with 1% SDS. The lysate was incubated overnight at 55°C with 0.5 mg/ml Proteinase K. The lysates were extracted first with phenol, then with phenol:chloroform:isoamyl alcohol (25:24:1) and then with chlorform:isoamyl alcohol (24:1). Genomic DNA was ethanol precipitated and spooled onto a capillary tube. Southern blots were performed as described (Stöger et al., 1993). Briefly, DNA was dissolved in 10 mM Tris-HCl pH 7.6, 1 mM EDTA (TE), digested with the restriction enzymes *Hpa*II and *Pvu*II, and separated on a 0.8% agarose gel. Southern blots were generated by standard methods (Sambrook et al., 1989) and filters were hybridized with an intron-specific probe covering region 2 of the *Igf2r* receptor (pPP4, see Stöger et al., 1993). A probe from region 1 of the *Igf2r* gene was used to check for complete digestion of genomic DNA (p*X*/*gf2r*, see Stöger et al., 1993). All Southern blots were repeated with at least two independent restriction digests of the genomic DNA.

PCR for Zfy

DNA from EG cell lines was analyzed by PCR for the presence of a Y chromosome. Primers used were 5′-AAGATAAGCTTA-CATAATCACATGGA and 3′-CCTATGAAATCCTTTGCTGCA-CATGT (Nagamine et al., 1989). PCR reactions were cycled at 94°C for 45 seconds, 62°C for 25 seconds and 72°C for 1 minute for 30 cycles. The presence of a Y chromosome was indicated by a 600 base pair reaction product.

Skeletal preparations

Skeletal preparations were performed essentially as described (McLeod, 1980). Briefly, newborn mice were first killed using Metofane, then incubated in distilled water at room temperature

overnight, scalded with hot water for thirty seconds, skinned and eviscerated. Cartilage was stained with Alcian Blue and bone counterstained with Alizerin Red. Skeletons were cleared with KOH and glycerin then stored in glycerin.

RESULTS

EG cell lines derived from C57BL/6 8.5 days p.c. PGCs can contribute to the germline of chimeric mice

Initial experiments from our laboratory to derive EG cells in culture used embryos from matings of ICR random bred females with (C57BL/6 x DBA)F_1 males (Matsui et al., 1991, 1992). Here, we have used the inbred mouse strain C57BL/6 to control for any genetic differences that may affect the derivation of EG cell lines. C57BL/6 blastocysts have been used previously to generate totipotent ES lines (Ledermann and Burki, 1991).

EG cell lines were first obtained from 8.5 days p.c. C57BL/6 embryos and early passage EG cells (passage number 6-10) were used for both blastocyst injection and genomic DNA isolation. Cell lines were also tested for the presence of a Y chromosome by performing PCR with primers specific for *Zfy* (see Methods). All cell lines were karyotyped and male cell lines with a normal karyotype were injected into BALB/c blastocysts. Table 1 shows the number of chimeras obtained from four of these EG lines. Male and female chimeras were bred to either BALB/c or ICR mice to test for the ability of the cells to differentiate into functional germ cells. The results of these matings are shown in Table 2. Male chimeras from two independent cell lines ($TGC^{8.5}10$ and $TGC^{8.5}19$) were able to transmit the C57BL/6 genome through the germline (Table 2; Fig. 1). No female chimeras gave birth to pigmented pups.

Generation of EG cell lines from gonadal PGCs

To ask whether PGCs become restricted in their ability to form EG cell lines after entering the gonad, we repeated these experiments with germ cells from later stage C57BL/6 embryos. Germ cells from the gonads of 15.5 days p.c. embryos and newborn mice did not give rise to EG cell lines under the conditions used previously. However, we were able to derive EG cell lines from the genital ridges of 12.5 days p.c embryos, at a time when most of the PGCs have migrated into them. At 12.5 days p.c. it is possible to identify the sex of the genital ridge by its morphology, so male and female genital ridges were pooled separately. The frequency of EG cell line formation is much lower with genital ridges from 12.5 days p.c. embryos compared to posterior tissue of 8.5 days p.c. embryos. From several separate experiments, ten cell lines were obtained from the equivalent of 6/100 of a genital ridge (approximately 432 PGCs, as calculated from Tam and Snow, 1981) as compared to more than 20 from the equivalent of the posterior portion of 1/6 of an 8.5 days p.c. embryo (approximately 24 PGCs, as calculated from Tam and Snow, 1981). All ten cell lines were derived from male gonads; no cell lines were obtained from female gonads. Two karyotypically normal cell lines were tested and shown to form differentiated tumors in nude mice and contribute to coat color chimeras. Table 1 shows the frequency of chimerism for these two EG cell lines ($TGC^{12.5}1$ and $TGC^{12.5}2$). The chimeras did not show a bias toward maleness: of the 9 surviving adult chimeras from both cell lines, there are 6 females and 3 males. These were bred to test for germline transmission but no pigmented pups resulted from these matings. Two of eleven identified chimeras from these two cell lines exhibited skeletal abnormalities and died soon after birth (within one to two days). The skeleton of one of these two newborns exhibited malformations of the rib cage and sternum (data not shown).

Table 2. Germline transmission of the EG cell lines $TGC^{8.5}10$ and $TGC^{8.5}19$

	Number of chimeras	Number of pups
$TGC^{8.5}10$ male chimeras transmitting only BALB/c genome	5 individuals	123 pups
$TGC^{8.5}10$ male chimeras transmitting both C57BL/6 and BALB/c genomes	Male #3 Male #12 Male #13 Male #14	1 of 71 pups pigmented 5 of 21 pups pigmented 1 of 32 pups pigmented 1 of 18 pups pigmented
$TGC^{8.5}19$ male chimeras transmitting only BALB/c genome	5 individuals	68 pups
$TGC^{8.5}19$ male chimeras transmitting both C57BL/6 and BALB/c genomes	Male #1	2 of 62 pups pigmented

Imprinting of the Igf2 receptor gene

In the somatic tissues of the adult mouse, the *Igf2r* gene is parentally imprinted with expression only from the maternal allele (Barlow et al., 1991). Recent work has identified a specific region in the *Igf2r* gene, region 2, within the second intron 27 kilobases downstream of the transcriptional start site of the gene, that is hypermethylated only on the maternally inherited allele (Stöger et al., 1993). This methylation pattern may be one factor regulating the transcription of *Igf2r*, as mice that possess two mutated copies of the methyltransferase gene

Table 1. Chimeras from blastocyst injection of EG cell lines

	Cell line	Passage number	Blastocysts injected	Pups born	Total chimeras	Chimeras not surviving
EG cell lines derived from 8.5 days p.c. embryos	$TGC^{8.5}10$	P6-12	181	63	21	5
	$TGC^{8.5}11$	P6-9	41	18	3	1
	$TGC^{8.5}12$	P8-10	62	36	3	3
	$TGC^{8.5}19$	P7-10	129	39	7	0
EG cell lines derived from 12.5 days p.c. genital ridges	$TGC^{12.5}1$	P6-13	193	94	8	2
	$TGC^{12.5}2$	P8-12	156	64	3	0

The contribution of these EG cells to the viable chimeras ranges from <5% to approximately 40% as judged by coat color.

Fig. 1. Germline transmission of TGC$^{8.5}$10. Male chimera from EG cell line TGC$^{8.5}$10. ICR random bred female, and pups from two separate litters. Pigmented pups (agouti and non-agouti) illustrate germline transmission of the C57BL/6 derived EG cell line.

display an altered methylation pattern in this region as well as downregulated expression of *Igf2r* (Li et al., 1993). Fig. 2 shows the methylation pattern of *Igf2r* region 2 for some of the EG cell lines tested. Half of the 8.5 days p.c. derived EG cell lines (9 out of 18), including TGC$^{8.5}$10, one of the two cell lines to transmit through the germline, show both a 3 kilobase and a 500 base pair hybridizing fragment implying that they carry one methylated and one non-methylated allele (Fig. 2C,D). This pattern of methylation is characteristic of somatic cells (Fig. 2A, mefs) and 5 different pluripotent ES cell lines (Fig. 2A for D3 and R1, and data not shown for J1, SC6 and TL1. The remaining cell lines, including TGC$^{8.5}$19, the other totipotent cell line, have a different pattern of methylation in which both alleles are unmethylated (Fig. 2C, D; Table 3) and show only a 500 base pair hybridizing fragment.

In order to evaluate the methylation pattern of *Igf2r* region 2 during PGC maturation, EG cell lines were established from PGCs at different times. EG cell lines from earlier PGCs (8.0 days p.c.) were generated and these cell lines were assayed for their methylation pattern. The results were similar to those with the EG cell lines derived from 8.5 days p.c. PGCs. Some cell lines (10 out of 18) showed both alleles to be unmethylated (for example TGC$^{8.0}$9, TGC$^{8.0}$10, TGC$^{8.0}$14, TGC$^{8.0}$15 and TGC$^{8.0}$16 in Fig. 2B; Table 3) whereas others (8 out of 18) showed a methylated and an unmethylated allele (for example TGC$^{8.0}$12 in Fig. 2B; Table 3). All ten EG cell lines derived from 12.5 days p.c. genital ridges showed an unmethylated pattern for region 2 (Fig. 2E).

Occasionally, when longer exposures of Southern blots were examined, some cell lines showed a faint 3 kb hybridizing fragment that was much less intense than the 3 kb fragment of

Table 3. The methylation status and sex of all EG cell lines

Cell line	Y chromosome	Methylation of region 2
EG cell lines derived from 8.0 days p.c. PGCs		
TGC$^{8.0}$1	+	met/unmet (partial)
TGC$^{8.0}$2	+	met/unmet (partial)
TGC$^{8.0}$3	+	met/unmet (partial)
TGC$^{8.0}$4	+	unmet
TGC$^{8.0}$5	+	met/unmet (partial)
TGC$^{8.0}$7	-	unmet
TGC$^{8.0}$8	+	unmet
TGC$^{8.0}$9	+	unmet
TGC$^{8.0}$10	+	unmet
TGC$^{8.0}$11	+	unmet
TGC$^{8.0}$12	-	met/unmet (somatic)
TGC$^{8.0}$13	+	met/unmet (somatic)
TGC$^{8.0}$14	+	met/unmet (partial)
TGC$^{8.0}$15	+	unmet
TGC$^{8.0}$16	+	unmet
TGC$^{8.0}$17	+	met/unmet (partial)
TGC$^{8.0}$18	-	unmet
TGC$^{8.0}$19	+	unmet
EG cell lines derived from 8.5 days p.c. PGCs		
TGC$^{8.5}$1	+	met/unmet (partial)
TGC$^{8.5}$2	+	met/unmet (partial)
TGC$^{8.5}$3	+	met/unmet (somatic)
TGC$^{8.5}$4	-	unmet
TGC$^{8.5}$5	-	met/unmet (partial)
TGC$^{8.5}$6	+	unmet
TGC$^{8.5}$8	-	unmet
TGC$^{8.5}$9	-	met/unmet (somatic)
TGC$^{8.5}$10	+	met/unmet (somatic)
TGC$^{8.5}$11	-	met/unmet (partial)
TGC$^{8.5}$12	-	met/unmet (partial)
TGC$^{8.5}$15	-	unmet
TGC$^{8.5}$16	-	unmet
TGC$^{8.5}$17	-	unmet
TGC$^{8.5}$18	-	unmet
TGC$^{8.5}$19	+	unmet
TGC$^{8.5}$21	+	met/unmet (somatic)
TGC$^{8.5}$22	-	unmet
EG cell lines derived from 12.5 days p.c. germ cells		
TGC$^{12.5}$1	+	unmet
TGC$^{12.5}$2	+	unmet
TGC$^{12.5}$3	+	unmet
TGC$^{12.5}$4	+	unmet
TGC$^{12.5}$5	+	unmet
TGC$^{12.5}$6	+	unmet
TGC$^{12.5}$7	+	unmet
TGC$^{12.5}$8	-	unmet
TGC$^{12.5}$9	+	unmet
TGC$^{12.5}$10	+	unmet
Other cells		
mouse embryo fibroblasts		met/unmet (somatic)
J1		met/unmet (somatic)
R1		met/unmet (somatic)
D3		met/unmet (somatic)
SC6		met/unmet (somatic)
TL1		met/unmet (somatic)

Footnote: Examples of the different methylation patterns can be seen in the following figures:
somatic pattern in Fig. 2A for ES cells and mefs
partial methylation pattern in Fig. 3A for TGC$^{8.5}$5
unmethylated pattern in Fig. 2D for TGC$^{8.5}$19.

either ES cells or fibroblasts. We interpret this as partial methylation of region 2, due to heterogeneity in the cultures. A summary of the methylation pattern of region 2 and the sex of the cell lines is presented in Table 3. There appears to be no correlation between the methylation pattern and the sex of the cells.

In order to test the possibility that the methylation pattern of region 2 of the *Igf2r* could change as ES and EG cell lines are grown in culture, several lines were cultured to late passage numbers (between 17 and 34). We have cultured ES cells to later passages (p=17) and have never seen alteration of the normal somatic pattern of methylation. Two of the three EG cell lines tested ($TGC^{8.5}19$ and $TGC^{12.5}8$) had both alleles of *Igf2r* unmethylated at early passage. As shown in Fig. 3, both of these cell lines maintained this methylation pattern in culture, even as late as passage 34. In contrast, $TGC^{8.5}5$, which had partial methylation at early passages (p=7, 11, 15), lost this methylation and the 3 kb hybridizing fragment by late passage numbers (p=20, 26, 29).

DISCUSSION

Derivation of EG cell lines by treating PGCs with growth factors

Previous work from our laboratory (Matsui et al., 1991,1992; Labosky et al., 1994) and others (Dolci et al., 1991; Godin et al., 1991; Resnick et al., 1992) and the data reported here show that the combination of SF, LIF and bFGF allows the establishment of EG cell lines. The mechanism by which this is achieved is not clear. One possibility is that this combination of growth factors somehow alters the developmental program of the PGCs so that they now behave like the epiblast or inner mass cells from which they were derived. Alternatively, the combination of growth factors may block the terminal differentiation of the PGCs, arresting them as an immature or precursor PGCs. At present the only difference that we have observed between EG cells and blastocyst-derived ES cells is the pattern of DNA methylation within region 2 of the *Igf2r* gene. Both cell types differentiate in vitro and in vivo, and both EG cells and ES cells can differentiate into functional sperm and thereby transmit their genome through the germline (Table 2; Matsui et al., 1992; Labosky et al., 1994; Stewart et al., 1994). Our results regarding the methylation pattern of region 2 of the *Igf2r* gene support the second alternative, that the EG cells resemble an immature germ cell rather than an inner cell mass cell, since most of the EG cell lines have a pattern of methylation more similar to that of late germ cells (Brandeis et al., 1993) than somatic cells and ES cells. Additionally, we have used our culture conditions with epiblasts from earlier embryos (6.5 days p.c.) and so far have been unable to establish stem cell lines, further supporting the theory that the EG cell lines derived from 8.0 and 8.5 days p.c. PGCs do not resemble epiblast cells.

Our data here suggest that PGCs from 8.5 days p.c. embryos and 12.5 days p.c. genital ridges are not equivalent in their response to the combination of SF, bFGF and LIF. Although pluripotent EG cell lines can be derived from both 8.5 and 12.5 days p.c. germ cells, the process is much more efficient with younger PGCs. This is illustrated by the observation that only ten cell lines were obtained from approximately four hundred 12.5 days p.c. PGCs while over twenty cell lines were obtained from an estimated twenty four 8.5 days p.c. PGCs. It appears that germ cells in 15.5 days p.c., newborn and 8 days postnatal

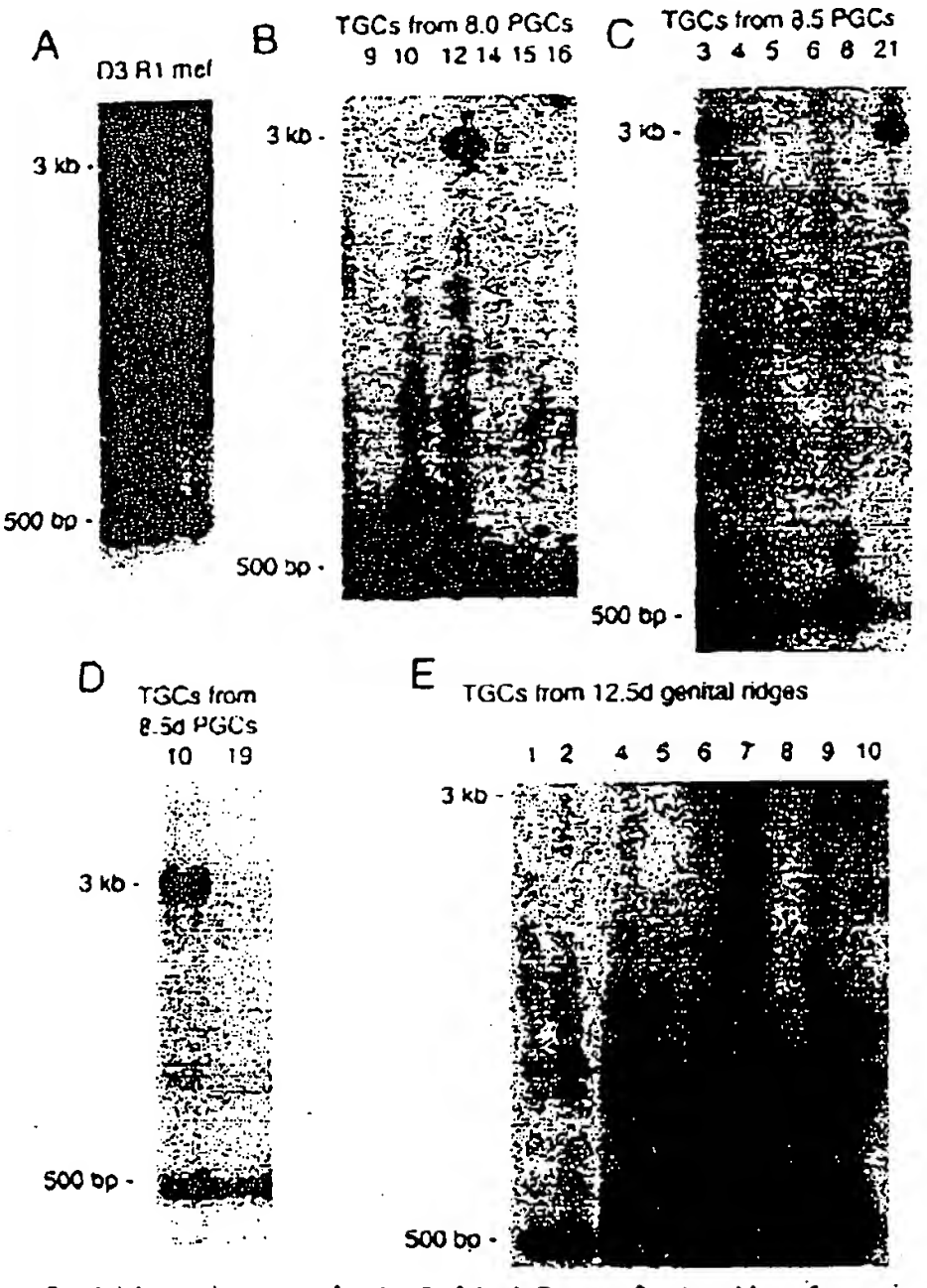

Fig. 2. Methylation status of region 2 of the *Igf2r* gene. Southern blots of genomic DNA from different cell lines digested with restriction enzymes *Hpa*II and *Pvu*II and hybridized with a probe for region 2 of the *Igf2r* gene. (A) D3 and R1, two different ES cell lines, and mouse embryo fibroblasts (mef). (B) EG cell lines derived from 8.0 days p.c. primordial germ cells. (C) EG cell lines derived from 8.5 days. p.c., primordial germ cells. (D) The two EG cell lines derived from 8.5 days p.c. primordial germ cells which transmitted through the germline. (E) EG cell lines derived from 12.5 days p.c. genital ridges.

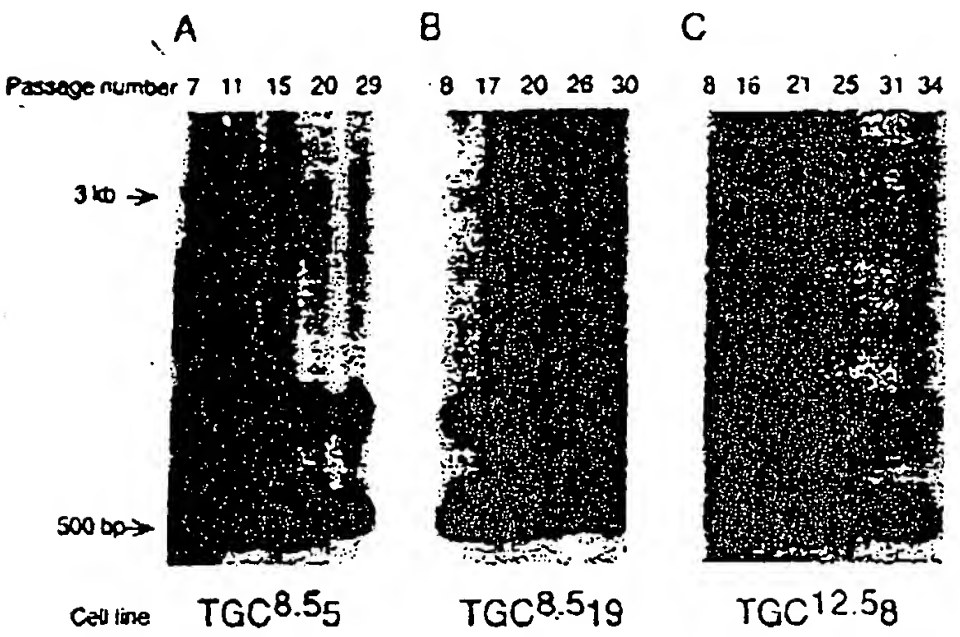

Fig. 3. Temporal changes in methylation status of region 2 of the *Igf2r* gene in EG cell lines. Southern blots of genomic DNA from different cell lines digested with restriction enzymes *Hpa*II and *Pvu*II and hybridized with a probe for region 2 of the *Igf2r* gene. (A) $TGC^{8.5}5$ at passage numbers 7, 11, 15, 20, 29; (B) $TGC^{8.5}19$ at passage numbers 8, 17, 20, 26, 30; (C) $TGC^{12.5}8$ at passage numbers 8, 16, 21, 25, 31, 34.

gonads do not respond to this combination of growth factors since we have been unable to establish EG cell lines from these stages with C57BL/6 inbred mice. Germ cells become postmitotic after they reach the genital ridge and it is possible that as they become quiescent they no longer respond to SF, LIF and bFGF perhaps because they downregulate one or more receptors for these factors. In support of this hypothesis, it has been shown (Manova and Bachvarova, 1991) that *c-kit*, the receptor for SF, is down regulated in PGCs shortly after they reach the genital ridge.

Our data raise the possibility that the EG cell lines derived from 12.5 days p.c. PGCs may be qualitatively different from the EG cell lines derived from 8.5 days p.c. PGCs. The EG cell lines derived from 12.5 days p.c. PGCs, while pluripotent, do not appear to be toupotent. A sex bias is not seen in chimeras after blastocyst injection and these resulting chimeras are less extensive than chimeras from EG cell lines derived from 8.5 days p.c. PGCs (as judged by coat color contribution) even when as many as 20 EG cells are used for each blastocyst injection (Table 2). Possible reasons for a qualitative difference are discussed below.

Imprinting of the Igf2r gene in EG cells

We have shown here that in approximately half of the EG cell lines derived from 8.0-8.5 days p.c. PGCs and all of the EG cell lines derived from 12.5 days p.c. genital ridges the methylation of region 2 of the *Igf2r* gene differs from normal somatic cells. Instead of the normal region 2 methylation pattern, with the maternal allele methylated and the paternal allelle unmethylated, these EG cell lines have both alleles unmethylated in the majority of the cells.

There are several possible explanations for these methylation differences. First, it may reflect the in vivo methylation status of the *Igf2r* gene in the founding PGCs. The mouse embryo undergoes massive general demethylation of genomic DNA at the 8- to 16-cell stage and a subsequent remethylation, which is completed by implantation (Monk et al., 1987). In germ cells, the general methylation changes are not identical to those seen in the rest of the embryo (Monk et al., 1987, Kafri et al., 1992). While the DNA in the somatic cells of the embryo has begun to be remethylated by 4.5 days p.c., germ cells remain relatively unmethylated at 12.5-13.5 days p.c.. By 15.5 days p.c. they are partially remethylated, with the process completed by 18.5 days p.c.. These observations on general demethylation of genomic DNA do not apply to region 2 of the *Igf2r* gene since the maternal-specific methylation of this region, directly inherited from the oocyte, is preserved through preimplantation (Stöger et al., 1993, Brandeis et al., 1993). However, region 2 does become unmethylated in both male and female germ cells by 13.5 days p.c. (Brandeis et al., 1993), implying that the genomic imprint for *Igf2r* is erased sometime between the blastocyst and 13.5 days p.c.. If the methylation pattern for *Igf2r* region 2 of our EG cell lines represents the methylation pattern of founding PGCs, the absence of methylation reported here in most EG cell lines reflects erasure of the *Igf2r* genomic imprint. Our results suggest that some PGCs have erased their imprint by 8.0-8.5 days p.c., with all having done so by 12.5 days p.c..

At present we cannot distinguish between an erased or an androgenetic imprint of the *Igf2r* gene (both alleles unmethylated), but our observations for the *Igf2r* gene may reflect a general absence or erasure of imprinting signals in the EG cells and not simply an androgenetic pattern. In support of this speculation, our results showing that EG cells from 8.5 days p.c. PGCs can form healthy chimeras and in some cases contribute to the germline illustrates that these EG cells differ from androgenetic ES cells (Mann et al., 1990; Mann and Stewart, 1991). However, we cannot eliminate the possibility that some of the EG cells from gonadal 12.5 days p.c. PGCs have acquired an androgenetic imprint since some of the skeletal defects of the chimeras obtained with these EG cells resemble the defects of androgenetic ES cell chimeras, although they are less severe and not as prevalent.

An alternative explanation for the unmethylated pattern in the EG cells is that methylation of *Igf2r* region 2 is not stable during in vitro culture. We have shown here that the somatic pattern of methylation seen in blastocyst-derived ES cells remains stable for up to 17 passages in culture. In addition, the unmethylated pattern in EG cells remains stable in several different lines for up to 34 passages. However, one EG cell line ($TGC^{8.5}5$) that displayed methylation at early passage (p=7, 11, 15) lost this methylation by later passages (p=20, 26, 29).

This observation implies that the methylation differences between different EG cell lines may not pre-exist before the lines were established but instead are the result of a difference in their response to in vitro culture. It is also possible that the methylation is unstable in EG cells because of their origin from PGCs. This instability may reflect an in vivo difference between PGC-derived EG cell lines and inner cell mass-derived ES cell lines, reflecting the difference in the developmental program of the founding cells. It is also noteworthy that, once unmethylated, the methylation of region 2 is not regained, implying that this erased pattern may be the normal end result for a PGC as it reaches the gonad.

The difference in the methylation pattern of region 2 in $TGC^{8.5}10$ and $TGC^{8.5}19$ (Fig. 2D) implies that the imprinted status of the *Igf2r* gene in an EG cell line does not affect its ability to pass through the germline. This result is perhaps not surprising in view of the fact that, although parthenogenetic embryos cannot form normal mice (Stevens, 1978; Clarke et al., 1988; and Fundele et al., 1990), it has been observed that parthenogenetic ES cells can contribute to chimeras and form functional germ cells giving rise to normal offspring (J. Mann and C. Stewart, unpublished data).

In conclusion, the two hypotheses that we have proposed for the differences in methylation of region 2 of the *Igf2r* gene between ES cells and EG cells may not be mutually exclusive. It is possible that the methylation differences between the various early passage EG cell lines reflect the changing methylation status of the migrating PGCs. It is also possible that the heterogeneity and plasticity of this methylation pattern in vitro is another property of PGCs. PGCs are programmed to erase their imprint in vivo and may be carrying out that program in vitro. In the future, the identification of specific regions such as region 2 in the *Igf2r* gene that regulate other imprinted genes will allow the generalities of these ideas to be explored.

We should like to thank Babru Samal, AMGEN, Inc. for recombinant LIF and SF. Drs. Jeff Mann and Chris Wright for helpful comments on the manuscript, and Linda Hargett for her excellent technical assistance. P.A.L. is an Associate and B.L.M.H. an Investigator of the Howard Hughes Medical Institute.

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Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J.,

DOCKET NO. 2200.007
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
BRIGID L. M. HOGAN)
) Group Art Unit: 1802
Serial No. 07/958,562)
) Examiner: D. Saunders
Filed: October 8, 1992)
)
For: "PLURIPOTENTIAL EMBRYONIC)
STEM CELLS AND METHODS OF)
MAKING SAME")

AMENDMENT

Honorable Commissioner of
Patents and Trademarks

Washington, D.C. 20231

NEEDLE & ROSENBERG, P.C.
Suite 1200
The Candler Building
127 Peachtree Street, N.E.
Atlanta, Georgia 30303-1811

January 17, 1995

Sir:

This amendment is in response to the July 15, 1994 Office Action. A request for a three-month extension of time is requested herewith.

IN THE CLAIMS

Please amend the claims as follows:

Cancel claims 1-4 and 20-22 without prejudice.

Claim 5, line 6: delete "cell" and substitute therefor --cells--.

A1 6 ~~10~~. (amended) A composition comprising a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of [embryonic ectoderm or] primordial germ cells.

A2 13 ~~19~~. (amended) A method of screening cells which can be promoted to become [an] pluripotent embryonic stem [cell] cells comprising contacting the cells with basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow proliferation of the cells and determining which cells become pluripotent embryonic stem cells.

Please add the following new claim:

A3 14 --~~25~~. (new) A composition comprising a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to promote the B formation ~~formulation~~ of pluripotent embryonic stem cells from ~~the~~ primordial germ cells.--

REMARKS

Claims 1-22 were pending in this application. Claims 1-4 and 20-22 have been canceled above in response to a restriction requirement. Applicant hereby reserves the right to prosecute the canceled claims in a Continuing Application. Claim 5 has been amended to correct a typographical error. Claim 10 has been amended to remove unnecessary alternative language. Claim 19 has been amended to properly state that the embryonic stem cells are pluripotent. The amendment to claim 19 is supported throughout the specification and is simply made for clarity. New claim 23 has been added. Claim 23 mirrors the language of claim 10 but emphasizes that the amount of the factors promotes "the formation of pluripotent embryonic stem cells from primordial stem cells." New claim 10 is likewise supported throughout the specification which describes the formation of pluripotent embryonic stem cells from primordial stem cells, see especially page 10, lines 7-10 and in the Example. No new matter is believed added by these amendments and entry is respectfully requested.

In the Office Action the Examiner initially restricted the claims to Groups I-IX. However, Groups II-VII were examined with Elected Group VIII because the claims could be searched without undue burden. Applicant hereby affirms the oral election of Group VIII. However, applicant traverses the continued

restriction of Groups I and IX. Applicant believes that examining Groups I and IX also does not present undue additional burden on the Examiner since all the claims arise out of the same invention. Therefore, withdrawal of the restriction is respectfully requested.

Applicant respectfully acknowledges the Examiner's time and courtesies shown to applicant in the October 28, 1994 interview. The interview was very helpful in clarifying the Examiner's concerns.

In view of the above amendments and following remarks reconsideration of the rejections and allowance of the pending claims is respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

The specification is objected to and claims 5, 6, 12, 13 and 15-19 stand rejected under 35 U.S.C. § 112, first paragraph. Specifically, the Examiner alleges that those skilled in the art would not expect that the methods described would be applicable to other species of animals. The Examiner cites Evans et al. in support of his proposition as allegedly teaching that ungulate stem cells have morphological characteristics different from those of rodents.

Applicant respectfully submits that the Examiner's position is not scientifically supported. Evans et al. concerns a very different procedure which cannot be extrapolated to the subject application.

Specifically, Evans et al. was concerned with a method of deriving ungulate embryonic stem cells from a blastocyst stage embryo. A blastocyst is a very early embryo which has not yet implanted onto the uterine wall. At the time of the Evans et al. patent publication it was known that other labs had tried very hard to isolate pluripotent embryonic stem cells from rat, pig and sheep without success. What seems to happen is that the inner cell mass cells differentiate very easily into endoderm and they fail to proliferate further as pluripotent embryonic stem cells. (In fact, there have been reports within the last year of success in obtaining cells from Rhesus monkey blastocysts and perhaps one recent success from rat blastocysts.) In view of the perceived problems isolating pluripotent stem cells from blastocysts and Evans et al.'s desire to obtain a patent on their ungulate stem cells, it was reasonable for Evans et al. to state that those skilled in the art would not expect that methods of obtaining stem cells from mice <u>blastocysts</u> may not be directly applicable to other species.

Applicant's claimed invention <u>does not</u> use blastocysts to derive embryonic stem cells. Applicant uses a very different

method to derive embryonic stem cells. As disclosed throughout the application and the Example, applicant's embryonic stem cells are derived from primordial germ cells dissected from post-implantation embryos.

Importantly, post-implantation embryos are very consistent between species as to the location of the primordial germ cells. The site at which primordial germ cells (PGCs) can be first detected (at the base of the allantois and yolk sac), their migration along the hind gut and their entry into the embryonic gonads is, based on the literature, highly conserved in mammalian embryos (see, for example, Hamilton and Mossman, *Human Embryology* 4th edition, pp. 401-403, The Macmillan Press Ltd., London (1972) (Exhibit A) and Moore and Persaud, *The Developing Human: Clinically Oriented Embryology* 5th edition, p. 281-283, W. B. Saunders Co., Philadelphia (Exhibit B), for human; Hendrickx and Sawyer, "Embryology of the Rhesus Monkey," *The Rhesus Monkey* Vol. II, Ed. G.H. Bourne, pp. 141-169, Academic Press, New York (1993)(Exhibit C), for Rhesus Monkey; and Schoenwolf, *Laboratory Studies of Vertebrate and Invertebrate Embryos*, 7th Edition, Prentis Hall, New Jersey (1995) (Exhibit D), for porcine). (Exhibits A through D, G and H will be submitted shortly with the original signed Declarations (Exhibits E and F) as a supplemental attachment.) This means that it is possible to predict with a high degree of confidence where to find primordial germ cells in mammalian embryos of different species at a given state of

embryonic development. Thus, one would reasonably expect success in different species using the claimed methods.

In view of these distinctions, it is clear that the concerns of Evans et al. simply do not apply to the claimed invention and the rejection under 35 U.S.C. § 112 should be withdrawn.

Applicant has attached hereto a signed facsimile copy of Declaration by Dr. Brigid L. M. Hogan (Exhibit E) setting forth data showing the isolation and proliferation of primordial germ cells from a post-implantation human embryo. The methods set forth in the Declaration to isolate human primordial germ cells are the same methods utilized for mice in the subject application with routine modifications based on the different age of the mouse and human embryos. (The original signed Declarations (Exhibits E and F) will be submitted shortly with Exhibits A through D, G and H as a supplemental attachment.)

In summary, because 1) the Evans et al. statements are not applicable to the post-implantation embryos used in the claimed methods or 2) the Declaration shows that applicant's method can be routinely applied to primordial germ cells of different species, the rejection under 35 U.S.C. § 112, first paragraph should be withdrawn.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 5 and 6 stand rejected under 35 U.S.C. § 112, second paragraph, as indefinite by referring to singular "cell" in line 6. "Cell" has been amended to cells in line 6. Thus, the rejection is deemed to be mooted.

Rejection under 35 U.S.C. § 102(a) or § 103

Claims 5-19 stand rejected under 35 U.S.C. § 102(a) or § 103 as allegedly obvious over Matsui et al. Attached hereto is a signed facsimile copy of a Declaration by Dr. Brigid L. M. Hogan (Exhibit F) stating that Yasuhisa Matsui and Krisztina Zsebo did not contribute to the conception of the claimed invention. (The original signed Declarations (Exhibits E and F) will be submitted shortly with Exhibits A through D, G and H as a supplemental attachment.) Specifically, Yasuhisa Matsui was a post doctoral student from Japan and only acted at Dr. Hogan's direction and supervision. Krisztina Zsebo only provided the steel factor at Dr. Hogan's request.

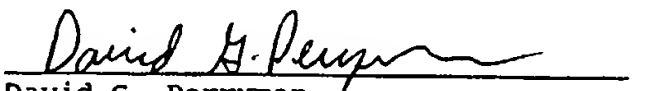
Therefore, the inventive entity is the same for Matsui et al. and the subject application and the rejection under 35 U.S.C. § 102(a) or § 103 should be removed.

As requested by the Examiner in the October 28, 1994 interview, copies of Sedivy and Joyner (1992) (cited on page 9, line 29) (Exhibit G) and Robertson (1987) (cited on page 11, line 25) (Exhibit H) will be submitted shortly as a supplemental attachment. As pointed out by the Examiner, these references demonstrate examples of utilities of the claimed invention.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending application is believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

A check in the amount of $435.00 is attached as required for the extension of time. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,



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Attorney Docket: 07265/102001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	FOR: HUMAN EMBRYONIC GERM CELL LINE
Gearhart *et al.*	
SERIAL NO.: 08/829,372	GROUP ART UNIT: 1651
FILED: March 31, 1997	EXAMINER: J. Weber

DECLARATION OF DR. MICHAEL SHAMBLOTT
UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Considered
30 Apr 99

Sir:

1. I, Michael J. Shamblott, declare and say I am a resident of Baltimore City, Maryland. My residence address is 2811 Halcyon Avenue, Baltimore City, Maryland 21214.

2. I received a Bachelor of Science degree in Biology from the University of Miami, Miami, Florida in 1986. I received a doctorate degree (Ph.D.) from the University of Maryland, Baltimore County in 1995. I am currently a Postdoctoral Fellow at Johns Hopkins School of Medicine, Department of Gynecology and Obstetrics, Division of Developmental Genetics. My curriculum vitae is attached, and indicates my expertise and experience in the areas of cellular and molecular biology.

3. I am a co-inventor of the subject matter claimed in the above-identified patent application. Those claims are set forth in Exhibit A.

4. I submit this declaration to provide facts known to me concerning the phenotype of the human embryonic germ cell subject matter claimed in my application as compared to the cells disclosed in the Hogan '357 and '926 patents, and particularly concerning the culture requirements of the cells for maintenance (secondary growth) following development of primary cultures.

5. The following experiments were performed by me or under my direction, using reagents and techniques according to the materials and methods described in the specification. To develop primary cultures, human embryonic germ (hEG) cells were isolated by dissecting genital ridges from 8-11 week LMP (last menstrual period) human aborted fetal material. The genital ridges were placed into approximately 0.5 ml of a conventional phosphate-buffered saline (PBS) solution. The tissue was then cut into small pieces, transferred to a conical tube and allowed to settle. A majority of the PBS was removed and 1 ml of 0.05% trypsin -0.53mM sodium EDTA was added to the tube. The tissue was then repeatedly pipetted through a 100 μl pipette tip to further disaggregate the cells.

Fifteen ml hEG growth medium (D-MEM, 4500 mg/l D-glucose, 2200 mg/l sodium bicarbonate) containing 15% embryonic stem cell-qualified fetal calf serum (BRL, Gaithersburg, MD); 2 mM glutamine (BRL); 1 mM sodium pyruvate (BRL); 1000-2000 U/ml human recombinant leukemia inhibitory factor (LIF, Genzyme, Cambridge, MA); 1 ng/ml human recombinant basic fibroblast growth factor (bFGF, Genzyme); and 10 μM forskolin in 10% DMSO was added to the cells. The tissue and cell suspension was then pelleted and the

hEG growth medium was removed. The cell pellet was resuspended in 3.2 ml hEG growth medium.

Approximately 0.2 ml of the cell suspension was then added to each of sixteen wells of a 96-well tissue culture plate that had been previously prepared with a sub-confluent layer of STO mouse fibroblasts. The STO fibroblast cell layer had been cultured for 3 days in hEG growth medium that was not supplemented with LIF, bFGF, or forskolin, and then irradiated with 5000 rads of gamma irradiation to induce mitotic arrest.

The hEG cells and STO mouse fibroblasts were cultured for 7-10 days in hEG growth medium, supplemented with LIF, bFGF and forskolin as described above, at 37^{0}C with 5% CO_2 at 90% humidity. Growth medium was freshly prepared and replaced daily.

On the 10th day of culture, the cells were trypsinized and cells from each well were passaged to individual wells of a 24-well culture dish that had been previously prepared with irradiated STO mouse fibroblasts (90% cells) and to individual wells of a 96-well tissue culture plate that had been previously prepared with irradiated STO mouse fibroblasts (10% cells).

6. To determine if hEG cultures required LIF and bFGF for maintenance, these components were omitted singly and in combination. On at least four occasions, primary cultures were divided and secondary (*i.e.*, maintenance) cultures were established using either fresh culture medium or medium from which LIF or bFGF or both was omitted. The secondary cultures where LIF, bFGF or both were omitted were not maintained (*i.e.*, failed to continue proliferating), in contrast to the cultures grown in the presence of LIF and bFGF (*i.e.*, normal

culture medium). These data fully support the existence of the phenotypic characteristics recited in the claims and the fundamental phenotypic differences of the claimed cells when compared to those disclosed in the Hogan '357 and '926 patents.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12 April, 1999
Date

Michael J. Shamblott, Ph.D.

86489.U1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James A. Thomson

Serial No.: 09/982,637

Filed: 10/18/2001

For: PRIMATE EMBRYONIC STEM CELLS

Date: October 19, 2004

Group Art Unit: 1632

Examiner: Joseph T. Woitach

Docket No.: 960296.97877

COMMENTS REGARDING REQUEST FOR INTERFERENCE

Commissioner for Patents
P O Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The applicant in the above-identified patent application has become aware that another patent applicant, the applicant in U.S. Serial No. 08/813,829 (the Hogan application) is seeking to have an interference declared between that application as one party and, as the other party, this application and its two parent cases, now U.S. Patents No. 6,200,806 and 5,843,780. Because the applicant here believes that it would be inappropriate for the Patent and Trademark Office to declare such an interference, and because this present patent application would be potentially subject to that interference, the applicant presents the comments that follow.

From a review of the record, there appears to be a misunderstanding, created by the applicant of the Hogan application in several respects. In the interests of brevity and communication the clearest reasons why the applicant here believes there is a misunderstanding, the applicant will limit its comments here to a single a factual question and single a legal question. The applicant here will have other objections to the Hogan application if an interference is declared, but for purposes of this paper, only these two issues are discussed. The factual question has to do with the differences between human embryonic stem cells (ES cells) and human embryonic germ line cells (EG cells). The legal question has to do with the applicability of 35 U.S.C. §135(b) on the request for an interference by the Hogan applicant.

Factual Issue

Turning first to the factual question. The applicant here wants to emphasize that EG cells and ES cells are not the same. ES cells and EG cells are distinct and have different capabilities and characteristics. The distinction between these two types of cells is apparent both from their source of origin and from the characteristics of those cells when grown in culture.

As the Examiner is probably aware, the origin of human embryonic germ cell lines derives from work done in the laboratory of Professor John Gearhart at the John Hopkins School of Medicine. That work resulted in a patent application which has generated at least three issued patents so far, U.S. Patents No. 6,090,622, No. 6,245,566, and No. 6,331,406. In the technology disclosed in those patents, the cells are created by the extraction of primordial germ cells from a post blastocyst human embryo. The derivation of the cells and characteristics of the cells are described in these patents in language that makes it clear that the Gearhart group actually created those cell lines in culture.

The present application here is a continuation of the previously issued and previously mentioned '780 and '806 patents to Dr. James Thomson of the University of Wisconsin. In the specification of this patent application, as well as its parent patents, the creation of primate embryonic stem cells, or ES cells, from cells cultured from the blastocysts of primates is specifically described. The applicant has further presented evidence that this method has been extended to the creation of human embryonic stem cells (Thomson, Science, 282 pp. 114-1147 Nov. 1998).

Note that patents exist on both the ES cells (Thomson) and the EG cells (Gearhart), but neither patentee has suggested that there should be, nor has there been, any interference between claims directed to those different cell types. This is because ES cells and EG cells are distinct and easily distinguishable from each other.

While there are many techniques that can be used to distinguish the cell types, for the purposes of this document, it is appropriate to refer specifically to the disclosures regarding these cells in the applicable patents. Primate (and human) ES cells are specifically reported in the specification of this application, and its parent patents, to be independent of, and insensitive to, the effect of a protein factor known as LIF (leukemia inhibitory factor). This is specified in the present specification (page 27, lines 6 to 19 and page 32, line 24 to page 33, line 77) and the specification of the '780 patent at column 16, lines 36-43. Primate (and human) ES cells are negative for a cell surface marker known as SSEA-1, and the attribute of

being negative for SSEA-1 is specifically claimed in Claim 3 of each of the '780 patent and the '806 patent and recited in this specification at page 8, line 12, page 22, line 11 to page 23, line 27.

By contrast, the Gearhart patents report that human EG cells are dependent on the application of LIF. The LIF dependence is specifically recited in column 5, lines 42-55 of the Gearhart '662 patent. Human EG cells are also positive for the cell surface marker SSEA-1, as specifically recited in column 14, lines 6-10 and Claim 11 of the '662 patent.

Thus from the technical description in the prior patents, there are established two classes of differences between EG cells and ES cells. One class of difference comes from the source or origin of the materials (inner cell mass of blastocysts for ES cells and primordial germ cells for EG cells) and the other distinguishing difference in characteristic has to do with the biochemical differences in the cells themselves (LIF dependence and SSEA-1 presentation). ES cells and EG cells are distinct and separate cell types.

The relevance of this observation to the question of whether there should be an interference between the Hogan application, and this application has to do with the fact that Hogan does not teach any methodology which could conceivably give rise to cells which we now refer to as ES cells. The specification of the Hogan application specifies as a starting material for the cultures to be created primordial germ cells from post blastocysts embryos. This is the same starting materials as the Gearhart EG cells.

Note that when Hogan published scientific papers about her cells, the very same cell cultures described in this patent application, Hogan referred to the cells as mouse EG cells. See, Labosky, Barlow and Hogan, *Development*, 120, pp 3197-3204 (1994), a copy of which is attached. Hogan specifically noted in that paper that her cells are "termed embryonic germ cells or EG cells to distinguish them from blastocyst-derived embryonic stem (ES) cells", *Id* at 3198.

In her original filing, Hogan by necessity restricted her claims to starting materials other than blastocysts, because the invention that Hogan originally claimed encompassed murine pluripotent cells, and the isolation of murine embryonic stem cells from blastocysts was in the prior art at the time Hogan filed. Thus, it is no surprise that Hogan has previously explicitly represented to the Patent and Trademark Office, in connection with the technology described in the Hogan specification, that applicant's invention "does not use blastocysts" (Response dated January 17, 1995 in Serial No. 07/958,562 now U.S. Patent No. 5,453,357, copy enclosed). Thus, Hogan has made it clear that the invention described in the Hogan

application does not involve the use of a blastocyst. The inner cell mass of the blastocyst is the only source so far demonstrated from which human embryonic stem cells, as we now define them, can be created.

Some confusion does arise from the fact that the Hogan specification refers to the cells created as embryonic stem cells. However, a fair reading of the specification of Hogan would reveal that, at best, Hogan would create EG cells, not ES cells. So Hogan has told the scientific world, in her paper. The starting material in the Hogan specification is recited as primordial germ cells. Hogan specifically reports that the cells obtained are SSEA-1 positive (page 18, line 29 of Hogan specification). We know true human ES cells are SSEA-1 negative. Hogan is either referring to EG cells or is fatally mis-descriptive of human ES cells.

The Hogan applicant has made the argument that she has somehow presented claims that are generic to EG cells and ES cells. This argument is specious. First, the Hogan application is actually enabling of nothing with regard to human cells. No human actual EG cell line or ES cell line is reported in the specification of the Hogan patent. In fact, Gearhart was able to obtain a patent on human EG cells because of deficiencies in the enablement of the Hogan application for exactly the EG cell type. Enclosed with this submission is a Declaration of Dr. Shamblott from the file history of U.S. Patent No. 6,090,822. In this Declaration, Dr. Shamblott demonstrates that the teaching of Hogan was wrong with regard to the procedures necessary to create human embryonic germ cells. The issue, in fact, was that LIF dependency is not taught by Hogan, and Dr. Shamblott's dcclaration states that LIF is required for human EG cells. This observation resulted in allowance of the Gearhart patents which recite, as a specific limitation in their claims, LIF dependency. The language in the Gearhart claims('622 patent) requires that the cells be dependent upon a ligand which combined to a receptor which can heterodymerize with glycoprotein 130. The ligand is specifically recited in the specification to be LIF.

Note that it is logically impossible for the claims of this application and claim to human EG cells to be deemed corresponding to the same count. The claims here in this application recite that the cells will proliferate in culture without LIF. The Shamblott Declaration establishes that human EG cells will not grow in culture without LIF, and that limitation is included in each claim of the Gearhart '622 patent. Cells can be LIF dependent or LIF independent, but no cell or cell type can be both.

To summarize the factual issue, EG cells and ES cells are very different. In spite of the nomenclature used, the only human cells that could be even arguably created from the teaching of the Hogan specification are EG cells. While the applicant here doubts that Hogan has enabled human EG cells, even if one assumed arguendo that Hogan enabled the creation of human EG cells, Hogan clearly does not enable anything which has the characteristics of human ES cells. This notwithstanding the fact that Hogan has used the language of embryonic stem cells in her specification, in contrast to her scientific paper. Merely using those words is not sufficient to enable. In fact, Hogan has specifically disclaimed the only technology known to be able to create human ES cells. To consider Hogan as enabling of human ES cells is a mistake of fact.

Legal Issue

As to the legal question earlier mentioned, the Hogan applicant is barred by the provisions of 35 U.S.C. §135(b) from presenting claims which would interfere this application, since those claims would also interfere with the '780 patent to the applicant here. The reason is that the Hogan applicant did not present an allowable claim even arguable patentably indistinct from the claims of both the present patent application and the applicant's issued '708 patent until an amendment filed February 28, 2001, a date more than one year after the issuance of the '780 patent, which issued on December 1, 1998.

Until February 28, 2001, all of the claims of the Hogan application were under rejection for 102(b) based on a reference to Pera et al., which described a prior art cell type known as human embryonic carcinoma cells, or human EC cells. Human EC cells were prior art to both of the applicants here, teachings of human EC cells dating from as early as 1984 (See column 3 of the '780 patent). The existence of human EC cells was prior art to the Thomson '780 and '806 patents as well as to all the Hogan patents and applications. The claims of this application, as well as the claims of the parent Thomson patents, distinguish human EC cells by means of a recitation in each of the claims that the cells have a normal karyotype, or are euploid. This limitation distinguishes the prior art human EC cells, which have abnormal karyotype, or are anaploid.

The Hogan applicant, who was clearly aware of the existence of the prior issued Thomson patents, added a limitation by amendment on February 28, 2001 to her claims which added language requiring the cells having "normal karyotype." This limitation was added in spite of the fact that in the Hogan specification there is no teaching whatsoever that human cells created by the method described would have normal karyotype. The only

reference that can be found in the application to karyotyping refers only to mouse cell cultures.

Nevertheless, the record is clear that Hogan did not present a claim with the "normal karyotype" limitation until February 28, 2001, more than one year after the issuance of the '780 patent. Hogan has argued that the '780 patent is not relevant since its claims recite primate embryonic stem cells rather than specifically human. However, as all parties would readily admit, humans are primates. No one has yet pointed to any patentably significant difference or distinction between the culture of non-human primate ES cells and primate human ES cells. The distinction has no patentable significance. The PTO has already taken the position that primates and humans are obvious variants itself when it insisted on a terminal disclaimer between the claims of the '780 and the '806 patent, which it did.

Hogan has admitted that her present claims are not patentably distinct from the claims of the '780 patent when she cited those claims as corresponding to the count proposed by Hogan for the interference. The test is the same. If the claims correspond, as Hogan has asserted that they do, then Hogan is barred by the provisions of §135(b).

The case law has made it clear that the presentation of a claim patentably indistinct from the claim of an issued patent more than one year after the issue date of the patent is absolutely barred by 35 U.S.C. §135(b). See, for example, *In re McGrew*, 120 F.3d 1263, 43 U.S.P.Q.2d 1632 (Fed.Cir. 1997) affirming *Ex parte McGrew*, 41 U.S.P.Q.2d 2004 (BPAI 1995). This case law is not equivocal. These claims are barred to Hogan.

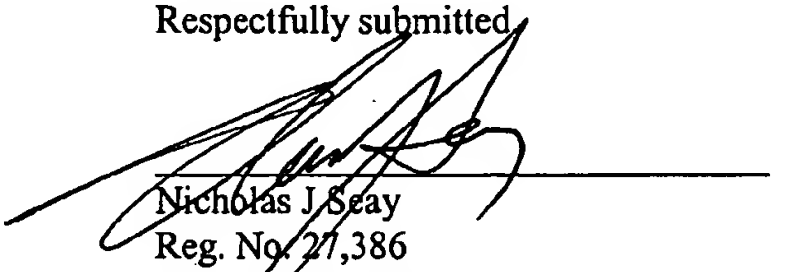
Note that although the claims of the '780 patent can be characterized as claims to a genus, and claims to a human ES cell culture can be characterized as a species, that is not enough by itself to avoid the application of §135 (b). While a genus and a species can be patentably distinct, they are not inherently patentably distinct. Here, there is no indication anywhere in the record that primate ES cells behave any differently or have any different distinguishing characteristics, from human ES cells. The test is whether the claims are to "the same or substantially the same subject matter" as the earlier patent. McGrew *supra*. Here the claims presented by Hogan are substantially the same subject matter as the claims to the '780 patent and are barred.

The applicant here wishes to note that it does not mean, by virtue of this filing, to concede that the Hogan specification is enabling of any type of human cell culture. The applicant believes Hogan does not enable any human pluropotent cell line at all. But these

observations are presented based on the understanding that the Examiner may, it appears, have some different belief.

Wherefore it is respectfully requested that these comments be considered in the decision by the Patent and Trademark Office as to whether or nót an interference be declared between the Hogan application and this one, or its parent patents. No interference is appropriate

Respectfully submitted,



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